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Inhibitor-Based Methods for Detection of Plasmid-Mediated AmpC β-Lactamases in *Klebsiella* spp., *Escherichia coli*, and *Proteus mirabilis*

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Non-beta-lactam inhibitor-based methods were evaluated for detecting plasmid-mediated AmpC β-lactamases in *Klebsiella* spp., *Escherichia coli*, and *Proteus mirabilis*. Using CLSI methodology and disks containing cefotetan alone and in combination with 400 μg of boronic acid, 9 of 10 positive control strains and 54 of 55 AmpC-PCR-positive clinical isolates were detected. Importantly 71% and 40% of these clinical isolates were susceptible by routine testing to ceftriaxone and ceftazidime, respectively. Boronic acid disks also enhanced detection of expanded-spectrum β-lactamases in AmpC producers.

Pai et al. recently demonstrated a high rate of clinical failure among patients who were infected in the bloodstream with AmpC-β-lactamase-producing *Klebsiella pneumoniae* and who received initial antimicrobial therapy, especially cephalosporin treatment (21). Of eight patients who were infected with organisms harboring DHA-1-like or CMY-1-like β-lactamases and who received initial and definitive therapy with cefotaxime or ceftazidime and no imipenem, six died. In contrast, 12 of 14 patients who were infected with organisms harboring one of the same two β-lactamases but who received imipenem as definitive therapy were cured. Clearly detection of AmpC-producing organisms is important to ensure effective therapeutic intervention and optimal clinical outcome. Imported *ampC* genes have also been found on the plasmids of other organisms that usually do not harbor these genes, such as *Klebsiella oxytoca*, *Escherichia coli*, and *Proteus mirabilis* (24). Some organisms may harbor plasmid-mediated expanded-spectrum β-lactamases (ESBLs) and AmpC β-lactamases.

Currently, no guidelines for detection of plasmid-mediated AmpC-producing organisms or organisms harboring multiple β-lactamases are available. A study was designed to detect AmpC β-lactamase-producing isolates of *Klebsiella* spp., *E. coli*, and *P. mirabilis* by methods that closely resemble the phenotypic confirmatory tests for ESBLs (4). Two novel methods that use boronic acid, a known class-C enzyme inhibitor (7), were first tested against positive and negative control strains and then applied as confirmatory tests to clinical isolates.

Positive control strains produced the following plasmid-mediated AmpC β-lactamases: MOX-1, LAT-2, DHA-1, DHA-2, ACC-1, MIR-1, ACT-1, FOX-1, and FOX-5b. This group included one or more enzymes from each of six families of plasmid-mediated AmpC β-lactamases recently selected by Perez-Perez and Hanson, who developed a PCR technique to identify family-specific *ampC* genes (23). Strains used as negative controls included an OMP F(-) strain, an OMP C(-) strain, and strains that produced the following β-lactamases: CTX-M-5, CTX-M-9, TEM-3, TEM-12, SHV-2, SHV-5, SHV-18 (*K. pneumoniae* ATCC 700603), OXA-3, and KPC-2. A K1-hyperproducing strain of *K. oxytoca* was also tested.

Clinical isolates were recovered at the McGuire Veterans Affairs Medical Center and identified by the Vitek and the API 20E systems (bioMerieux Vitek, Hazelwood, Mo.). Isolates were screened for cefoxitin susceptibility by the standard disk diffusion method using a 30-μg disk (Becton Dickinson Microbiology Systems[BDMS], Cockeysville, Md.) (20), and a total of 271 nonrepeat *Klebsiella* spp. ($n = 128$), *E. coli* ($n = 115$), and *P. mirabilis* ($n = 28$) isolates demonstrated zone diameters that were less than 18 mm. Screen-positive isolates were tested for the presence of the AmpC β-lactamase by a three-dimensional method and by tests that use boronic acid. For the three-dimensional method, a standard cefoxitin disk was placed on Mueller-Hinton agar that was inoculated with one of two *E. coli* strains (ATCC 25922 or ATCC 11775), and a slit was cut in an outward radial direction and filled with 30 μl of a 5 McFarland cell suspension of test organism (5). Enhanced growth of the surface organism at the point where the slit intersected the zone of inhibition was considered a positive test result. Isolates positive by the three-dimensional method were tested by isoelectric focusing of cell extracts (5), and if no enzyme band was seen after application of a cloxacillin-impregnated filter paper to the focused gel, multiplex PCR was performed (23). AmpC-PCR-positive isolates were tested for ceftazidime and ceftriaxone susceptibility by the Vitek system and for ESBLs by the double-disk potentiation method (6).

Disks containing boronic acid were prepared as follows: 120 mg of phenylboronic acid (benzeneboronic acid; Sigma-Aldrich, Milwaukee, Wis.) was dissolved in 3 ml of dimethyl sulfoxide. Three milliliters of sterile distilled water was added to this solution. Twenty microliters of the stock solution was dispensed onto disks containing 30 μg of cefotetan or blank disks (BDMS). Disks were allowed to dry for 30 min and used immediately or stored in airtight vials with desiccant at 4 and at

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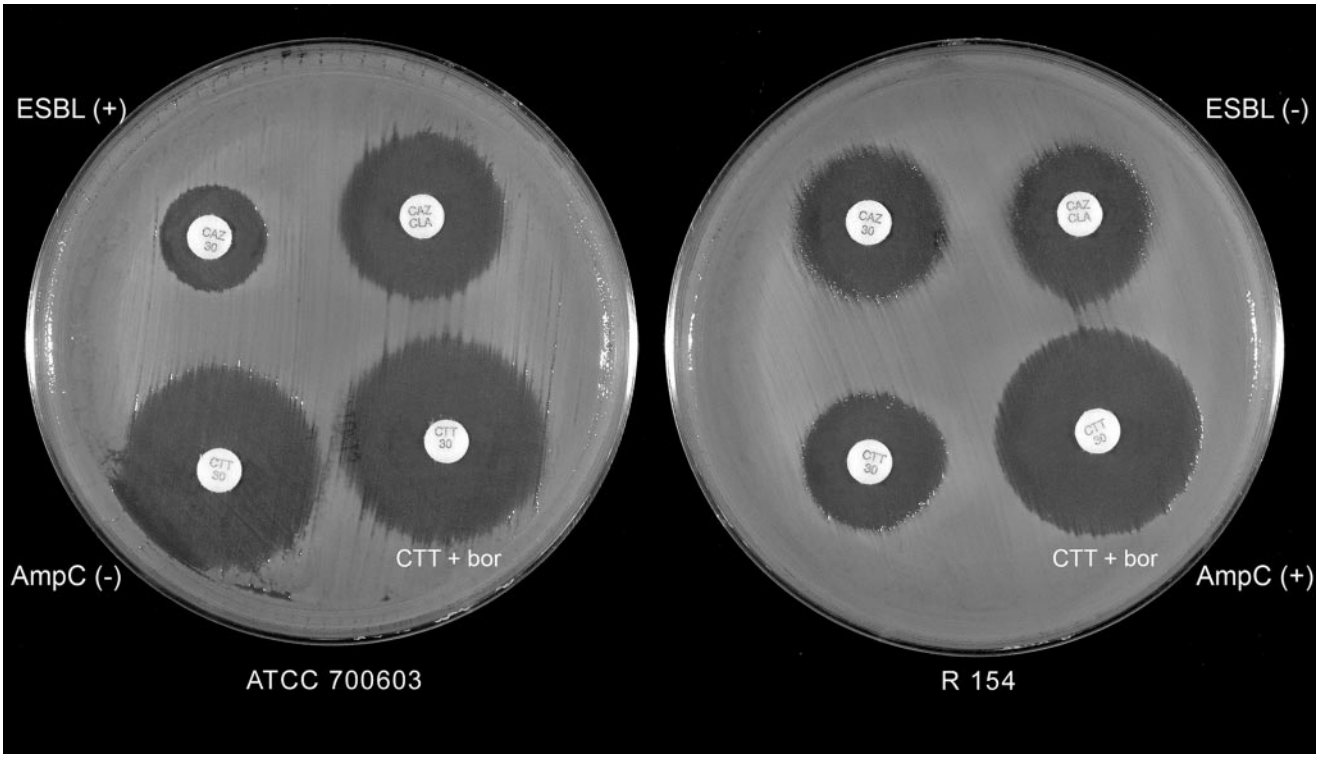

FIG. 1. Comparison of the standard confirmatory disk test for ESBL and the boronic acid (bor) disk test for AmpC β-lactamase with two *K. pneumoniae* control strains. Strain ATCC 700603 produced ESBL and no AmpC β-lactamase, and strain R154 produced AmpC β-lactamase and no ESBL. CAZ, ceftazidime; CAZ CLAV, ceftazidime and clavulanic acid; CTT, cefotetan; CTT + bor, cefotetan and 400 μg of boronic acid.

−70°C. The boronic acid disk test was performed by inoculating Mueller-Hinton agar (Remel, Lenexa, Kans.) by the standard disk diffusion method (20) and placing a disk containing 30 μg of cefotetan (BDMS) and a disk containing 30 μg of cefotetan and 400 μg of boronic acid onto the agar. Inoculated plates were incubated overnight at 35°C. An organism that demonstrated a zone diameter around the disk containing cefotetan and boronic acid that was 5 mm or greater than the zone diameter around the disk containing cefotetan was considered an AmpC producer.

For the boronic acid broth test, MICs for cefotetan and for cefotetan in combination with 400 μg of boronic acid per ml were determined by the microdilution technique using Mueller-Hinton broth (BDMS) and a standard inoculum of 5×10^5 CFU/ml (19). Trays were incubated at 35°C for 16 to 20 h. MIC results of control strains *E.* coli ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were within the expected ranges. An eightfold or greater concentration decrease in the MIC of cefotetan tested in combination with boronic acid, versus the MIC for cefotetan when tested alone, was considered indicative of AmpC production.

Figure 1 shows the growth patterns of two *K. pneumoniae* control strains with the boronic acid disk test. The *K. pneumoniae* ATCC 700603 is an ESBL producer, and the difference in the zone size between the disk containing ceftazidime and the disk containing ceftazidime and clavulanic acid is evident. This strain harbors no AmpC, and the zone sizes of disks containing cefotetan and cefotetan plus boronic acid were the same. In contrast, strain R154 harbors no ESBL but produces a FOX AmpC β-lactamase. For this strain the zone size of the disk containing ceftazidime and clavulanic acid was slightly smaller (2 mm) than the zone of the disk containing ceftazidime, probably because of AmpC induction by clavulanic acid. However, the zone size of the disk containing cefotetan and boronic acid was 9 mm greater than the zone size of the disk containing cefotetan alone. Some enhanced clearing is visible near the side of the cefotetan and the ceftazidime and clavulanic acid disks that are closest to the disk containing boronic acid.

Table 1 shows that all the positive and negative control strains, with the exception of R181, were accurately detected by the boronic acid tests. Strain R181 harbors ACC-1, which is known to hydrolyze cephamycins poorly relative to other AmpC β-lactamases and was susceptible to cefoxitin and cefotetan by current breakpoint criteria (1). Disks stored at 4 and at −70°C were stable for 10 and 20 weeks, respectively.

Of a total of 271 screen-positive clinical isolates, 55 isolates were AmpC-PCR-positive, and the boronic acid disk test detected 54 of the isolates in this latter group (13 *Klebsiella*, 38 *E. coli* and 3 *P. mirabilis*) (Table 2). The broth test failed to detect five plasmid-mediated AmpC producers (3 *E. coli* and 2 *P. mirabilis*), and for this reason, the disk test is the recommended method. It is unclear why the disk test missed one of the AmpC-producing *P. mirabilis* isolates, although it may be due to swarming phenomena, which is often seen with this organism on agar media. MacConkey agar did not improve the test results. This is the first report of a DHA-like AmpC β-lactamase in *P. mirabilis*.

TABLE 1. Results of cephamycin tests with positive and negative control strains

Strain	Organism	Resistance mechanism	FOX DD (mm)[a]	3-dm[b]	Boronic acid tests		Reference
					Difference in zone diam[c]	Decrease in MIC[d]	
R185	*K. pneumoniae*	MOX-1	6	+	12 (+)	128 (+)	16
R155	*E. coli*	LAT-2	6	+	12 (+)	64 (+)	11
R170	*K. pneumoniae*	DHA-1	6	+	14 (+)	128 (+)	9
R182	*K. pneumoniae*	DHA-2	6	+	13 (+)	64 (+)	8
R181	*E. coli*	ACC-1	24	+	4 (−)	4 (−)	18
R156A	*E. coli*	MIR-1	6	+	13 (+)	64 (+)	22
MCV-4	*K. pneumoniae*	ACT-1	6	+	13 (+)	64 (+)	2
R154	*K. pneumoniae*	FOX-1	10	+	9 (+)	32 (+)	13
M621	*K. pneumoniae*	FOX-5b	6	+	9 (+)	64 (+)	Hanson et al.[e]
R183	*E. coli*	OMP F(−)	22	−	1 (−)	≤1 (−)	15
R184	*E. coli*	OMP C(−)	26	−	0 (−)	≤1 (−)	15
R165	*K. oxytoca*	Hyper K1	24	−	2 (−)	≤1 (−)	12
R188	*E. coli*	CTX-M-5	22	−	0 (−)	≤1 (−)	10
R189	*E. coli*	CTX-M-9	20	−	1 (−)	≤1 (−)	26
R140	*E. coli*	TEM-3	19	−	0 (−)	≤1 (−)	28
R141	*E. coli*	TEM-12	19	−	1 (−)	≤1 (−)	31
R142	*K. pneumoniae*	SHV-2	23	−	2 (−)	≤1 (−)	17
R145	*E. coli*	SHV-5	29	−	2 (−)	≤1 (−)	14
ATCC 700603	*K. pneumoniae*	SHV-18	13	−	1 (−)	2 (−)	25
R173	*E. coli*	OXA-3	19	+/−	1 (−)	2 (−)	30
R169	*K. pneumoniae*	KPC-2	20	+	4 (−)	4 (−)	27

[a] Cefoxitin (FOX) zone diameter by standard disk diffusion (DD) method.
[b] Three-dimensional (3-dm) test result. +, positive; −, negative.
[c] Difference between the zone diameter of the disk containing cefotetan plus boronic acid and that of the disk containing cefotetan. Values are in millimeters. The test interpretation is shown in parentheses.
[d] *n*-Fold decrease in the MIC of cefotetan tested in combination with boronic acid versus the MIC of cefotetan tested alone. The test interpretation is shown in parentheses.
[e] N. D. Hanson, P. E. Coudron, E. S. Moland, and C. C. Sanders, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1458, 1999.

It should be noted that unlike *Klebsiella* spp., *E. coli* carries a chromosomal gene for AmpC β-lactamase, which is normally expressed in negligible amounts but may be increased due to alterations in AmpC regulatory genes (3). The boronic acid tests are unable to distinguish between *E. coli* isolates that produce AmpC β-lactamase due to imported genes and isolates with altered AmpC chromosomal genes. This may explain why the number of *E. coli* isolates that were positive by the boronic acid tests (Table 2) was greater than the number that were positive by PCR.

Chromosomal AmpC may also explain in part, the relatively high number of *E. coli* isolates that were positive by the three-dimensional test (Table 2). However, five *Klebsiella* isolates also yielded false-positive results by this test. Interestingly, four of these nonAmpC-producers were *K. pneumoniae* isolates that produced ESBLs. Other investigators have reported that cefoxitin resistance in general was a nonspecific indicator of AmpC production (29).

The susceptibility test results of ceftriaxone and ceftazidime for PCR-positive isolates underscore the importance of detecting AmpC-producing organisms (Table 2). Overall, 71% and 40% of these isolates were susceptible by automated testing to ceftriaxone and ceftazidime, respectively, including all 13 *Klebsiella* spp. that were susceptible to ceftriaxone. Cephalosporins

TABLE 2. Test results for screen-positive isolates[a]

Organism (*n*)	No. of isolates														
	3-dm		Boronic acid tests[b]				PCR[c]			CAZ[d]			CRO[d]		
			Disk		MIC										
	Neg	Pos	Neg	Pos	Neg	Pos	FOX	CIT	DHA	S	I	R	S	I	R
K. pneumoniae (122)	106	16	110	12 (12)	110	12 (12)	12			3 (25)	6 (50)	3 (25)	12 (100)		
K. oxytoca (6)	4	2	5	1 (1)	5	1 (1)	1					1 (100)	1 (100)		
E. coli (115)	22	93	40	75 (38)	39	76 (35)	8	30		15 (40)	15 (40)	8 (21)	22 (58)	10 (26)	6 (16)
P. mirabilis (28)	24	4	25	3 (3)	26	2 (2)		3	1	4 (100)			4 (100)		
Total (271)	156	115	180	91 (54)	180	91 (50)	21	33	1	22 (40)	21 (38)	12 (22)	39 (71)	10 (18)	6 (11)

[a] Abbreviations: 3-dm, three-dimensional test; Neg, negative; Pos, positive; CAZ, ceftazidime; CRO, ceftriaxone; S, susceptible, I, intermediate; R, resistant.
[b] Values in parentheses are numbers of isolates positive by both boronic acid and PCR tests.
[c] FOX, CIT, and DHA are nucleotide groups, as described in reference 23. A total of 55 isolates were positive by PCR.
[d] Values are numbers of PCR-positive isolates that were susceptible, intermediate, or resistant to the drug, as determined by the Vitek system. Values in parentheses are percentages.

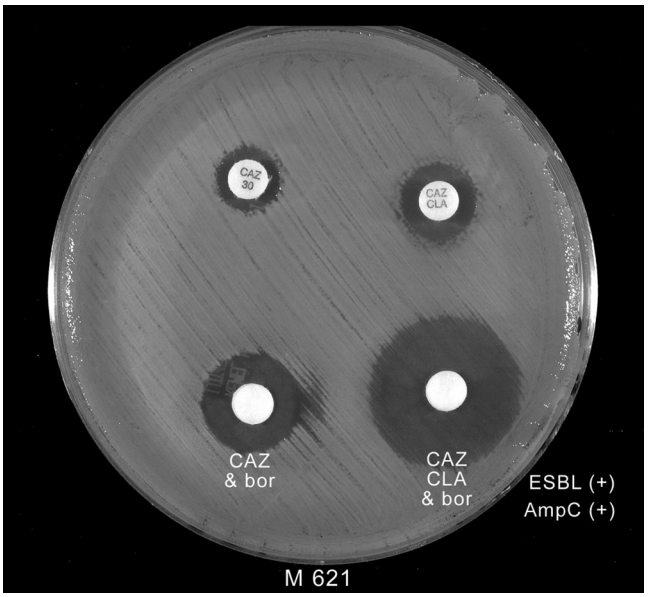

FIG. 2. Comparison of the confirmatory ESBL test using the standard ceftazidime and ceftazidime/clavulanic disks and the same two disks, each with a second disk, containing 400 μg of boronic acid (bor), placed on top. The *K. pneumoniae* isolate harbored both an ESBL and an AmpC β-lactamase.

are the preferred substrates for AmpC β-lactamases, and despite apparent in vitro susceptibility, the reported test interpretation for AmpC-producing isolates should be resistant for all cephalosporins. These organisms have been associated with treatment failure (21), and it is important that these steps be taken to ensure appropriate therapeutic intervention and improved infection control. The issue of positive boronic acid test results that are due to production of chromosomal AmpC in *E. coli* warrants further study.

One *K. oxytoca* and two *K. pneumoniae* isolates harbored both ESBL and AmpC β-lactamases. Figure 2 shows the growth pattern of one of these isolates when a second disk containing 400 μg of boronic acid [alone] was placed on top of the ceftazidime and the ceftazidime/clavulanic acid disks that are routinely used for ESBL testing (4). Twenty microliters of saline was delivered to the top of each second disk. Whereas when the standard procedure was used, the zone difference between the disks with and without clavulanic acid was only 2 mm, the zone difference between the same two disks, each with the second disk on top, was 7 mm. The AmpC enzyme masked detection of the ESBL.

In summary, the boronic acid disk test is a practical and efficient method that uses current CLSI methodology to detect plasmid-mediated AmpC β-lactamase in organisms that usually do not harbor genes for these enzymes. Investigators have demonstrated that patients who had bloodstream infections with these organisms and who were treated with expanded-spectrum cephalosporins had poor clinical outcome. However, in the current study many AmpC-producers were susceptible to expanded-spectrum cephalosporins by routine susceptibility tests. Therefore, isolates that are positive by the boronic acid disk test should be reported as being resistant to all cephalosporins. A boronic acid disk also enhances detection of isolates that harbor both ESBLs and AmpC β-lactamases.

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